Journal of Molecular Diagnostics, Vol. 7, No. 5, November 2005

Sensitive Detection of Polyalanine Expansions in *PHOX2B* by Polymerase Chain Reaction Using Bisulfite-Converted DNA

Hidekazu Horiuchi,* Ayako Sasaki,† Motoki Osawa,* Kazuki Kijima,† Yukiko Ino,‡ Ryoji Matoba,‡ and Kiyoshi Hayasaka†

From the Departments of Experimental and Forensic Pathology and Pediatrics,† Yamagata University Faculty of Medicine, Yamagata; and the Department of Legal Medicine,‡ Course of Social Medicine, Osaka University Graduate School of Medicine, Osaka, Japan*

Congenital central hypoventilation syndrome, also known as Ondine's curse, is characterized by idiopathic abnormal control of respiration during sleep. Recent studies indicate that a polyalanine expansion of *PHOX2B* is relevant to the pathogenesis of this disorder. However, it is difficult to detect the repeated tract because its high GC content inhibits conventional polymerase chain reaction (PCR) amplification. Here, we describe a bisulfite treatment for DNA in which uracil is obtained by deamination of unmethylated cytosine residues. Deamination of DNA permitted direct PCR amplification that yielded a product of 123 bp for the common 20-residue repetitive tract with replacement of C with T by sequencing. It settled allele dropouts accompanied by insufficient amplification of expanded alleles. The defined procedure dramatically improved detection of expansions to 9 of 10 congenital central hypoventilation syndrome patients examined in a previous study. The chemical conversion of DNA before PCR amplification facilitates effective detection of GC-rich polyalanine tracts. *(J Mol Diagn 2005, 7:638–640)*

Congenital central hypoventilation syndrome (CCHS), also known as Ondine's curse (MIM: 209880), is characterized by idiopathic abnormal control of respiration with an onset at birth or in infants.[1] Although CCHS patients breathe normally when awake, hypoventilation with shallow breathing occurs during sleep. Hypercapnia and hypoxia caused by apnea are life threatening unless the patient is under intensive clinical care. Amiel and colleagues[2] presented evidence that the paired-like homeobox 2B (*PHOX2B*) gene is the predominant disease locus in CCHS. The disease exhibits *de novo* mutations and autosomal dominant inheritance caused by triplet expansions of a stable 20-residue polyalanine repeat encoded in exon 3 of the gene. The prevalence of polyalanine expansions, ranging from 5 to 13 residues, in patients with CCHS has been reported.[3–5] In a previous study, heterozygous polyalanine triplet expansions were detected in 4 of 10 affected Japanese patients.[6]

Amino acid tandem repeats, also known as homopolymeric tracts, are abundant in the human genome. These tracts commonly include high contents of G and C.[7] The relevant region of *PHOX2B*, spanning 160 bp, which contains the triplet repeat along with its vicinity, also contains a high content of GC (≈88%). The unusually high GC content interferes with direct polymerase chain reaction (PCR) amplification, except for that of relatively large fragments more than 380 bp.[2,4] Even when PCR does operate, the smaller repeat allele is predominant, allowing the possibility that the other fully expanded allele does not undergo successful amplification.[4,8] To avoid technical difficulties arising from high GC contents, a bisulfite treatment for DNA has been developed for G- and C-containing repeat expansions such as that in fragile X syndrome.[9,10] This chemical deamination by bisulfite converts cytosine selectively to uracil on single-stranded DNA, on which conventional PCR potentially operates. This article describes our development of an analogous PCR protocol using deaminated DNA. It effectively amplifies polyalanine expansions of *PHOX2B* in CCHS patients in the previous study.[6] We subsequently re-examined the potential involvement of CCHS caused by the repeat expansion in forensic cases of sudden infant death syndrome (SIDS).[11]

Materials and Methods

Patients and DNA Preparation

DNA was prepared from intracardiac blood of 48 SIDS patients obtained at autopsy as well as the peripheral blood of 190 unrelated Japanese healthy volunteers, aged 20 to 56 years. Specimens from all 10 CCHS cases and 42 SIDS cases were described in previous reports.[6,11] It was uncer-

Supported by the Uehara Memorial Foundation, Tokyo, Japan.

Accepted for publication May 10, 2005.

Address reprint requests to Motoki Osawa, M.D., Ph.D., Department of Experimental and Forensic Pathology, Yamagata University Faculty of Medicine, 2-2-2 Iida-nishi, Yamagata 990-9585, Japan. E-mail: mosawa@med.id.yamagata-u.ac.jp.

tain whether changes of triplets were detected sufficiently by the previous procedures. For the reason, these samples were reanalyzed in this series of experiments. Written informed consent was obtained from the patients' parents before experimentation. The institutional ethical committee approved the protocol of this study.

DNA Deamination

Bisulfite processing of DNA was performed as described by Clark and colleagues[12] with some modifications. Briefly, 1 μg of genomic DNA in a volume of 50 μl was denatured by adding freshly prepared NaOH to a concentration of 0.3 mol/L for 5 minutes at 95°C. The bisulfite reaction was performed at final concentrations of 2.5 mol/L sodium bisulfite and 0.5 mmol/L hydroquinone (Sigma-Aldrich, St. Louis, MO) at 55°C for either 2 or 12 hours under mineral oil. A Wizard DNA clean-up kit (Promega, Madison, WI) was used to purify the modified DNA. After purification, samples were desulfonated with NaOH at a concentration of 0.3 mol/L for 15 minutes at 37°C, then neutralized by the addition of 10 mol/L ammonium acetate. The DNA was ethanol-precipitated and finally dissolved in 40 μl of distilled water for PCR analysis.

PCR Amplification

The polyalanine repeat region of the *PHOX2B* gene (GenBank accession no: NT_016297) was amplified using the oligonucleotide primer set of 5′-AGGTGAATTTGGTAAGGGTGG-3′ (mPHF1) and 5′-ACCCAACCTTATCCAAAACCC-3′ (mPHR2), according to the C to T exchanged sequence of the unmethylated DNA. In addition to 1.0 μl of the modified DNA solution, the PCR reaction was performed in a total volume of 15 μl containing 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of dNTP, and 1 U of HotStar *Taq*DNA polymerase (Qiagen, Hilden, Germany). No supplemented Q solution for GC-rich sequences was added to the reaction mixture. The amplification program was as follows: 95°C for 15 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 30 seconds. The PCR products were subsequently fractionated in 10% polyacrylamide gels and stained with ethidium bromide. The products were excised and reamplified when band shifts occurred. After agarose gel electrophoresis, the products were purified using a spin column (QIAEX II gel extraction kit; Qiagen). Direct sequencing was performed with a Big Dye Terminator cycle sequencing kit in an ABI 310 automated sequence analyzer (Applied Biosystems, Foster City, CA). The PCR products were also subcloned into p3T vector (MoBiTec, Goettingen, Germany) with T-A interaction.

Results and Discussion

Bisulfite causes hydrolytic deamination of cytosine residues in DNA to uracil residues, except for those at methylated CpG sites.[12] To reduce the high GC content of the polyalanine tract in *PHOX2B*, we incubated DNA with sodium bisulfite for either 2 or 12 hours at 55°C in this series of experiments. Because DNA double strands lose their complementarity after deamination, oligonucleotide primers can be designed from either modified strand. For convenience, strand-specific primers, designated mPHF1 and mPHR2, were made for the coding strand. Using the converted DNA as a template, a single product of 123 bp was obtained for the common 20-residue homozygotes by PCR in the absence of dimethyl sulfoxide, as expected (Figure 1). Uracil derivatives are amplified as T by DNA polymerase, thereby replacing the original C with T during the amplification. Consequently, direct sequencing identified the original sequence despite the mixture of T and C at unmethylated C sites. As shown in Figure 2, the replacements were more clearly visible after subcloning. A longer incubation time of 12 hours was required to obtain complete conversion at 55°C. Nevertheless, 2-hour incubation was sufficient to yield the PCR products in a time-efficient manner. An experiment addressing the subcloned fragments determined that ~60 to 70% conversion of C to T by sequencing after a 2-hour incubation. Because the polyalanine repeat consisted of four synonymous codons, rather than a single component, the mutated part is judged from the obtained sequence.

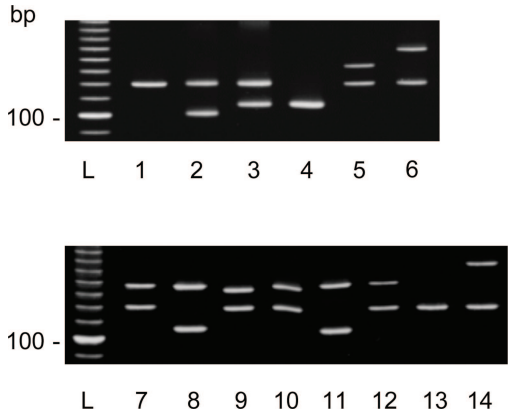

Figure 1. Polyacrylamide gel electrophoresis of typical PCR products obtained for the polyalanine tract of *PHOX2B* using the deaminated DNA as a template. The gel is stained with ethidium bromide. **Lane L**, 10-bp ladder; **lane 1**, homozygous 20-residue repeat; **lane 2**, heterozygous 13-residue repeat; **lane 3**, heterozygous 15-residue repeat; **lane 4**, homozygous 15-resiude repeat; and **lanes 5** to **14**, the 10 CCHS cases corresponding to those in the previous analysis.[5]

Among the specimens of 10 CCHS patients examined, 9 specimens (90%) were shown to carry heterozygous ex-

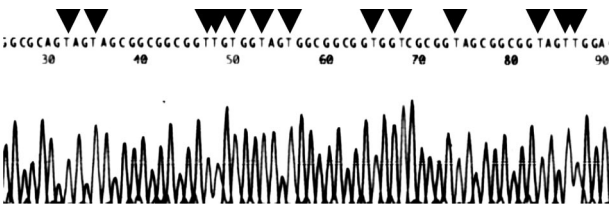

Figure 2. Nucleotide sequence of the converted DNA at the common 20-residue polyalanine tract. The sequence of a subcloned fragment is shown to demonstrate the replacement of C to T more clearly. **Arrows** indicate the converted T after bisulfite treatment.

pansions of 25-, 26-, 27-, 30-, and 33-residue repeats (n = 2, 3, 2, 1, and 1, respectively) after analysis using the improved procedure (Figure 1), in which the detection had been incapable for five specimens in the previous approach.[6] Furthermore, equal amplification of alleles was obtained. Incomplete detection in the previous approach may result from allele dropouts due to insufficient amplification of the fully expanded alleles. The other one had a frame-shift mutation. Ultimately, all clinically diagnosed CCHS patients were determined as carrying mutations of the *PHOX2B* gene. This result was consistent with observations by Weese-Mayer and colleagues,[3] by Matera and colleagues,[4] and Trochet and colleagues,[5] who detected mutations with high incidences of 65 (97%) of 67 CCHS cases, 25 (93%) of 27 cases, and 174 (93%) of 188 cases, respectively. We infer that the majority of clinically diagnosed CCHS are caused by mutations of *PHOX2B*.

To investigate the possibility that undetected CCHS is involved in cases of SIDS and healthy individuals, we re-examined the bisulfite-converted DNA from 48 SIDS patients, from 5 days to 12 months of age (mean age ± SD, 4.9 ± 3.5 months), and 190 unrelated patients.[11] In addition to the stable 20-residue repeat, contracted products of 13- and 15-residue repeat were evident, consistent with previous observations.[2–5] However, no expansions were encountered in both groups. Weese-Mayer and colleagues[13] have reported that no expansions were evident in 97 SIDS cases. These results strongly suggested that CCHS is not closely related to the SIDS etiology. In addition, the 15-residue repeat exhibited a common polymorphism with a frequency of 0.03 in the Japanese population group. One young female who exhibited a homozygote of 15-residue repeat appeared normal without respiratory symptoms.

The homopolymeric tract of polyalanine in homeobox transcription factors is responsible for many neurological malformations and disorders in which expanded variants are pathogenic.[14,15] Polyalanine expansions of *PHOX2B*, which transcriptionally controls neuronal differentiation, generate CCHS via dysfunction of the autonomic nervous system.[16] For that reason, the mutated variants need to be detected precisely. The unusually high GC content of the tract and its vicinity is refractory to conventional PCR reactions, even after the addition of dimethyl sulfoxide and 7-deaza-dGTP. It also remains a possibility of allelic imbalance to diploid during amplification.[4] As an elegant circumvention for analysis of GC-consecutive tracts, Weinhäusel and colleagues[9,10] applied DNA deamination to molecular diagnosis of fragile X syndrome, a CGG repeat, and Unverricht-Lundborg disease, a GC-composed dodecamer repeat. The bisulfite treatment used in this study was initially developed to detect methylation at CpG sites on genomic DNA.[12] Bisulfite selectively deaminates unmethylated cytosine residues, thereby weakening the complementary interaction between G and C. This phenomenon conceivably diminishes the inhibitory effect of the formation of secondary structures during PCR amplification. With regard to the amplified region in our study, the conversion of C to T reduced the calculated melting temperature (T_m) considerably, from 93 to 72°C.

In conclusion, in this series of experiments using converted DNA, small products less than 170 bp were obtained for the repeat expansions with no apparent allelic imbalance, which offered the advantage that the variants' sizes were precisely determined by subsequent electrophoresis. Moreover, the original sequence can be identified reliably by replacing T with C in the obtained sequence.

References

1. Weese-Mayer DE, Berry-Kravis EM: Genetics of congenital central hypoventilation syndrome: lessons from a seemingly orphan disease. Am J Respir Crit Care Med 2004, 170:16–21
2. Amiel J, Laudier B, Attie-Bitach T, Trang H, de Pontual L, Gener B, Trochet D, Etchevers H, Ray P, Simonneau M, Vekemans M, Munnich A, Gaultier C, Lyonnet S: Polyalanine expansion and frameshift mutations of the paired-like homeobox gene PHOX2B in congenital central hypoventilation syndrome. Nat Genet 2003, 33:459–461
3. Weese-Mayer DE, Berry-Kravis EM, Zhou L, Maher BS, Silvestri JM, Curran ME, Marazita ML: Idiopathic congenital central hypoventilation syndrome: analysis of genes pertinent to early autonomic nervous system embryologic development and identification of mutations in PHOX2b. Am J Med Genet 2003, 123A:267–278
4. Matera I, Bachetti T, Puppo F, Di Duca M, Morandi F, Casiraghi GM, Cilio MR, Hennekam R, Hofstra R, Schober JG, Ravazzolo R, Ottonello G, Ceccherini I: PHOX2B mutations and polyalanine expansions correlate with the severity of the respiratory phenotype and associated symptoms in both congenital and late onset central hypoventilation syndrome. J Med Genet 2004, 41:373–380
5. Trochet D, O'Brien LM, Gozal D, Trang H, Nordenskjold A, Laudier B, Svensson PJ, Uhrig S, Cole T, Niemann S, Munnich A, Gaultier C, Lyonnet S, Amiel J: PHOX2B genotype allows for prediction of tumor risk in congenital central hypoventilation syndrome. Am J Hum Genet 2005, 76:421–426
6. Sasaki A, Kanai M, Kijima K, Akaba K, Hashimoto M, Hasegawa H, Otaki S, Koizumi T, Kusuda S, Ogawa Y, Tuchiya K, Yamamoto W, Nakamura T, Hayasaka K: Molecular analysis of congenital central hypoventilation syndrome. Hum Genet 2003, 114:22–26
7. Alba MM, Guigo R: Comparative analysis of amino acid repeats in rodents and humans. Genome Res 2004, 14:549–554
8. Osawa M, Kaneko M, Horiuchi H, Kitano T, Kawamoto Y, Saitou N, Umetsu K: Evolution of the cystatin B gene: implications for the origin of its variable dodecamer tandem repeat in humans. Genomics 2003, 81:78–84
9. Weinhäusel A, Haas OA: Evaluation of the fragile X (FRAXA) syndrome with methylation-sensitive PCR. Hum Genet 2001, 108:450–458
10. Weinhäusel A, Morris MA, Antonarakis SE, Haas OA: DNA deamination enables direct PCR amplification of the cystatin B (CSTB) gene-associated dodecamer repeat expansion in myoclonus epilepsy type Unverricht-Lundborg. Hum Mutat 2003, 22:404–408
11. Kijima K, Sasaki A, Niki T, Umetsu K, Osawa M, Matoba R, Hayasaka K: Sudden infant death syndrome is not associated with the mutation of PHOX2B gene, a major causative gene of congenital central hypoventilation syndrome. Tohoku J Exp Med 2004, 203:65–68
12. Clark SJ, Harrison J, Paul CL, Frommer M: High sensitivity mapping of methylated cytosines. Nucleic Acids Res 1994, 22:2990–2997
13. Weese-Mayer DE, Berry-Kravis EM, Zhou L, Maher BS, Curran ME, Silvestri JM, Marazita ML: Sudden infant death syndrome: case-control frequency differences at genes pertinent to early autonomic nervous system embryologic development. Pediatr Res 2004, 56:391–395
14. Amiel J, Trochet D, Clement-Ziza M, Munnich A, Lyonnet S: Polyalanine expansions in human. Hum Mol Genet 2004, 13:R235–R243
15. Brown LY, Brown SA: Alanine tracts: the expanding story of human illness and trinucleotide repeats. Trends Genet 2004, 20:51–58
16. Cross SH, Morgan JE, Pattyn A, West K, McKie L, Hart A, Thaung C, Brunet JF, Jackson IJ: Haploinsufficiency for Phox2b in mice causes dilated pupils and atrophy of the ciliary ganglion: mechanistic insights into human congenital central hypoventilation syndrome. Hum Mol Genet 2004, 13:1433–1439